Regular Article

PLATELETS AND THROMBOPOIESIS

Functional platelet defects in children with severe chronic ITP as tested with 2 novel assays applicable for low platelet counts

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Key Points

- Pediatric chronic ITP patients with a severe bleeding phenotype exhibit functional platelet defects.
- The platelet microaggregation test and the platelet reactivity assay are able to assess platelet function at extremely low platelet count.

Immune thrombocytopenia (ITP) is an autoimmune disease with a complex heterogeneous pathogenesis and a bleeding phenotype that is not necessarily correlated to platelet count. In this study, the platelet function was assessed in a well-defined cohort of 33 pediatric chronic ITP patients. Because regular platelet function test cannot be performed in patients with low platelet counts, 2 new assays were developed to determine platelet function: first, the microaggregation test, measuring in platelets isolated from 10 mL of whole blood the platelet potential to form microaggregates in response to an agonist; second, the platelet reactivity assay, measuring platelet reactivity to adenosine diphosphate (ADP), convulxin (CVX), and thrombin receptor activator peptide in only 150 μL of unprocessed whole blood. Patients with a severe bleeding phenotype demonstrated a decreased aggregation potential upon phorbol myristate acetate stimulation, decreased platelet degranulation following ADP stimulation, and a higher concentration of ADP and CVX needed to activate the glycoprotein IIbIIIa complex compared with patients with a mild bleeding phenotype. In conclusion, here we have established 2 functional tests that allow for evaluation of platelet function in patients with extremely low platelet counts (<10^9). These tests show that platelet function is related to bleeding phenotype in chronic ITP. (*Blood*. 2014;123(10):1556-1563)

Introduction

Platelets play a critical role in hemostasis. When the vascular endothelium is disrupted, platelets adhere to subendothelium and initiate primary hemostasis. Excessive bleeding can occur if primary hemostasis is abnormal, either because of deficient platelet number or function. In vivo, primary hemostasis can be tested via bleeding time. However, this test does not distinguish between the varieties of causes of disturbed primary hemostasis.[1-3] This can be tested more specifically in vitro, but current methods require relative high numbers of platelets and are consequently unsuitable for patients with low platelet counts.[1,2,4]

Immune thrombocytopenia (ITP) is the most common cause of primary thrombocytopenia in children, with an incidence of ~1 in 20 000 children.[5,6] Although the pathophysiology of ITP is not fully understood, 2 major forms are recognized: acute ITP and chronic ITP. Acute ITP is characterized by a sudden onset of bruising and bleeding in an otherwise healthy child. Often there is a history of viral illness in the weeks preceding the onset of bruising.[5] Full blood counts show low platelet numbers (frequently <20 × 10^9/L) as the only abnormality. In acute ITP, autoantibodies, recognizing glycoproteins (GPs) on the surface of platelets and megakaryocytes, are considered the underlying cause.[7,8] These antibodies are thought to result in accelerated clearance of platelets and megakaryocytes and thereby may also lead to decreased production of platelets.[7,9] In chronic ITP, the attribution of autoantibodies to the pathogenesis of thrombocytopenia is less clear.

In the majority of pediatric ITP patients, thrombocytopenia resolves spontaneously within weeks or months. In ~25% of the patients, thrombocytopenia persists and becomes chronic.[5,10,11] During chronic ITP, platelet counts can vary in time from very low (<10 × 10^9/L) to almost normal. However, the observed bleeding tendency does not correlate strictly with platelet count. Cases with either low platelet counts without bleeding or relatively high platelet counts with severe bleeding do occur. Causes for this variation in bleeding tendency are unknown. We hypothesize that variation in platelet function can account for the differences observed in bleeding phenotypes. Until now it has not been possible to predict if an individual child with chronic ITP is at risk for severe bleeding attributable to platelet malfunction, because of the lack of reliable tests for platelet function in patients with low platelet counts.[2-4]

We here describe 2 functional platelet tests that can be used on patient material with very low (≥10 × 10^9/L) platelet numbers. The

The online version of this article contains a data supplement.

first test, the platelet microaggregation test, based on a recently developed test,[12] was adjusted to test platelet function directly by determining the potential of patients' platelets to form microaggregates together with platelets from a healthy control in response to an agonist. Ristocetin and phorbol myristate acetate (PMA) were used to activate platelets through the von Willebrand factor and the fibrinogen binding site on GPIbIX and GPIIbIIIa, respectively.[12-14] In the second assay, the platelet reactivity assay, the reactivity in 3 major physiological platelet activation pathways was determined in unprocessed whole blood by flow cytometry on the level of individual platelets.[15] Adenosine diphosphate (ADP), convulxin (CVX), and thrombin receptor activator peptide (TRAP) were used to activate platelets via P2Y receptors ($P2Y_1$ and $P2Y_{12}$), GPVI receptor, and proteinase-activated receptor 1, respectively. Furthermore, the platelet activation test quantifies both degranulation (P-selectin expression on the platelet surface) and activation of GPIIbIIIa (binding of fibrinogen to platelets). Both assays require a minimum of blood compared with classical functional platelet assays (ie, 10 mL of whole blood for the platelet microaggregation test and 150 µL of whole blood for the platelet reactivity assay). After validating both tests for low platelet numbers with platelets from healthy controls, the tests were used within a well-defined cohort of children with chronic ITP, and results were correlated with reported bleeding scores. Our results show that patients suffering from serious bleeding have impaired functional platelet capacities compared with patients with no or mild bleeding and healthy controls. Both tests yield valuable information that may be used to predict future bleeding tendencies in chronic ITP patients.

Methods

Patients

Children aged 6 to 13 years with chronic ITP were included in this multicenter observational study. Parents and patients aged 12 years and older gave written informed consent. The study was approved by the Institutional Review Board of the University Medical Center Utrecht and performed in accordance with the Declaration of Helsinki.

Chronic ITP was defined as isolated thrombocytopenia with a platelet count of $<100 \times 10^9$/L for more than 12 months. Patients were classified as having either a mild grade 0 to 3 or a severe grade 4 to 5 bleeding phenotype, according to the Buchanan bleeding score.[16] For scoring, all bleeding problems during the course of ITP were taken into account, irrespective of platelet count. These chronic patients were scored severe if they received grade 4/5 on the Buchanan scale based on both their worst bleeding event and recurrence of at least 1 more episode of grade 4/5 bleeding needing medical intervention. All except 1 of the severe patients also experienced a grade 4/5 in the 2 months prior to inclusion.

Data on duration of the ITP, bleeding tendency, and medication were collected by questionnaire and from the patients' medical files. All patients were tested for the presence of autoantibodies with indirect platelet immunofluorescence tests (PIFTs) and indirect monoclonal antibody immobilization of platelet antigens (MAIPA).

Blood obtained from adult healthy individuals served as positive controls, and 2 Glanzmann thrombasthenia patients were included as negative controls for the platelet microaggregation tests. Acquired Glanzmann patients have α-GPIIbIIIa autoantibodies, which inhibit platelet aggregation via the GPIIbIIIa route, whereas other pathways are normal. Primary Glanzmann patients lack GPIIbIIIa almost completely, but other pathways may also be hampered.[12,17]

Platelet microaggregation test

For the platelet microaggregation assays, peripheral whole blood was collected into a 10-mL BD Vacutainer with 17 IU sodium heparin (Becton, Dickinson, Plymouth, United Kingdom) and centrifuged for 15 minutes at

$218g$ to obtain platelet-rich plasma (PRP). PRP was washed 1:1 in sequestrin buffer (17.5 mM Na_2HPO_4, 8.9 mM Na_2EDTA, 154 mM NaCl, pH 6.9, 0.1% weight to volume ratio bovine serum albumin) and centrifuged for 6 minutes at $2374g$. Platelets were washed again by adding 10 mL sequestrin buffer and finally dissolved in 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) medium enriched with 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 5 mM glucose. To measure aggregation function, patients' platelets were suspended in HEPES medium to a final concentration of 10×10^6/mL; a minimum of 400 µL was required. Control platelets, from a healthy donor, were suspended in HEPES medium to a final concentration of 90×10^6/mL; a minimum of 800 µL was required. The platelets from the patients were stained with 0.8 µM PKH-26 (PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling [PKH26GL]; Sigma-Aldrich), whereas the control platelets were stained with 0.2 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) (C1157; Invitrogen). Both were incubated in the dark for 15 minutes at room temperature. Staining was stopped by adding citrate-phosphate-dextrose plasma from pooled AB^+ blood from healthy donors (20% final concentration). Subsequently, 500 µL 10×10^6/mL patient platelets were mixed with 500 µL 90×10^6/mL control platelets and incubated with 20 µM H-D-Phe-Pro-Arg-chloromethylketone (PPACK) (cat. 520222; Merck Biochemicals) for 5 minutes at 37°C while shaking at 300 rpm. Platelet mixes were incubated for 5 minutes with 3 mM $CaCl_2$ to enable platelet activation. A healthy control was always tested next to a patient (positive control). Platelets were activated with 100 ng/mL PMA (SC-3576; Santa Cruz) or with 1.5 mg/mL ristocetin (cat. 50705; Biopool, Trinity Biotech) to activate GPIIbIIIa and GPIbIX, respectively. Samples without an agonist served as a negative control. Samples were taken and fixed in 0.5% formaldehyde (Buffered Formaldehyde 4%; Klinipath, Duiven, The Netherlands) in a V-bottom 96-well plate (Nunc; Thermo Fisher Scientific), at 0, 5, and 10 minutes. Fixed samples were measured on a fluorescence-activated cell sorter (FACS) Canto II+HTS (BD Biosciences, San Jose, CA) and analyzed with BD Facs DIVA 6.1 software (BD Biosciences), and aggregation was calculated as follows: Aggregation (%) = (# aggregates [$CFSE^+PKH^+$ events])/(# patients' platelets [PKH^+ events] $\times 100\%$).

Platelet reactivity assay

Blood was collected into a 4.5-mL BD Vacutainer with 0.5 mL sodium citrate 3.2% (Becton, Dickinson). Serial dilutions of ADP (Roche, Almere, The Netherlands) to stimulate the P2Y receptors (starting from 125 µM), CVX (Pentapharm, Basel, Switzerland) to stimulate the GPVI receptor (starting from 39 ng/mL), and TRAP (Bachem AG, Bubendorf, Switzerland) to stimulate the proteinase-activated receptor 1 (starting from 625 µM), all in 8 fourfold dilutions, were prepared in a mixture of 47.5 µL HEPES buffered saline (consisting of 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$, and 5 mM KCl, pH 7.4, filtered through a 0.22-µm filter), 2 µL R-phycoerythrin (RPE)–labeled mouse anti-human P-selectin antibodies (#555524; BD Biosciences, Franklin Lakes, NJ), and 0.5 µL Alexa Fluor 488–labeled fibrinogen (Invitrogen, Eugene, OR).

A control sample, only containing 47.5 µL HEPES buffered saline, 2 µL RPE-labeled mouse anti-human P-selectin antibodies, and 0.5 µL Alexa Fluor 488–labeled fibrinogen, was prepared to determine platelet basal activation levels.

To measure patient and healthy control platelet reactivity, 5 µL fresh, citrate anticoagulated whole blood was added to all samples. After 20 minutes of incubation, 50 µL Optilyse B (Beckman Coulter Inc., Fullerton, CA) was added to fix the samples. After 10 minutes, 395 µL distilled water was added to lyse the erythrocytes. After half an hour of incubation at room temperature, the samples were kept at 4°C until analysis on the FACS Canto II flow cytometer from BD Biosciences, which was at all times performed within 24 hours. Single platelets were gated based on forward scatter (FSC) and side scatter (SSC) properties; 10 000 single platelets were measured in each sample. The median fluorescence intensity (MFI) of RPE-labeled mouse anti-human P-selectin antibodies and Alexa Fluor 488–labeled fibrinogen on platelets was measured with FACS analysis, representing the quantity of P-selectin and open GPIIbIIIa receptor per platelet.

The obtained FACS data were quantified using BD FACSDiva software 6.1.2. The platelet responsiveness to agonists was qualified by calculation of

Table 1. Baseline characteristics

	Mild bleeding phenotype (n = 23)	Severe bleeding phenotype (n = 10)	P
Age at inclusion, years*	10 (6-13)	12 (8-15)	.576
Age at diagnosis, years*	3 (2-9)	4 (2-12)	.773
Sex, male†	6 (26)	3 (30)	1.000
Platelet count at day of inclusion, $\times 10^9$/L*	74 (53-117)	58 (14-156)	.805
MPV at day of inclusion, fL*	9.4 (8.6-11.6)	10.8 (8.4-14.8)	.216
Positive indirect PIFT†	2 (9)	1 (10)	1.000
Positive indirect MAIPA†	4 (17)	2 (20)	1.000

MPV, mean platelet volume.

*Data represent median (interquartile range); P was calculated using Mann-Whitney U test.

†Data represent number (percentage); P was calculated using Fisher's exact test.

the 50% effective concentration (EC50) and the maximal response using GraphPad Prism 5.03 (GraphPad Software, San Diego, CA). The EC50 represents the agonist concentration generating a response halfway between baseline and maximum response. The response of the platelets to the highest agonist concentration in the dilution series represents the maximal effect of stimulation.

To validate the assay for use at low platelet count, PRP from healthy donors was isolated by centrifugation for 15 minutes at 160g, and platelet-poor plasma was obtained by centrifugation twice for 10 minutes at 2000g. Platelet number was set at 250, 50, 25, and 10×10^9/L by diluting PRP by addition of platelet-poor plasma. Five microliters of these mixtures was added to ADP, CVX, and TRAP samples to determine platelet reactivity at different platelet numbers.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 20.0.0 for Windows (IBM Corporation, Armonk, NY). Data are shown as median with interquartile range unless otherwise indicated. Wilcoxon signed ranks tests were performed to analyze data from related samples used in the validation experiments. For analysis of patient data, comparison between 2 groups was performed by Mann-Whitney U test for numerical data and by Fisher's exact test for categorical data. Correlation of 2 numerical variables was performed by Spearman's rank correlation. P values lower than .05 were considered to be statistically significant.

Results

Baseline characteristics

A total of 33 patients, all outpatients at Dutch university medical hospitals, were included in this study. As such, these patients represent a subgroup of ITP patients with increased bleeding phenotype. Ten patients were classified as having a severe bleeding phenotype (30.3%), and 23 patients as having a mild bleeding phenotype (69.7%) (Table 1). Platelet reactive autoantibodies were found in a minority of patients: 3 were found positive by indirect PIFT, and 6 by indirect MAIPA. These patients were equally distributed between groups.

Validation of 2 new functional platelet assays at low platelet count

For the microaggregation test, platelets were gated based on their FSC/SSC characteristics (Figure 1A). After mixing PKH26-labeled patient samples with CFSE-labeled control platelets, a time-dependent increase in aggregation was observed, with a maximal aggregation after

10 minutes. Aggregation was defined as events double positive in PKH and CFSE and quantified as shown in Figure 1B. We first determined the optimal and the lowest platelet number required for reliable results, by testing a range of proportions in healthy controls (supplemental Figure 1; see the *Blood* Web site). This was accomplished by changing the concentration of PKH-labeled platelets, whereas CFSE-labeled platelets were kept constant at 90×10^6/mL using healthy control platelets. A ratio of ~1 PKH26 (test platelet) to 10 CFSE-labeled control platelets was considered reliable and feasible, indicating that 10 mL whole blood with only 10×10^9 platelets per L was sufficient. We also crossed platelets from different healthy controls, to assess whether well-functioning platelets from 1 donor will reach a similar aggregation level when aggregated with platelets from different healthy controls. Similar aggregation levels were reached (supplemental Figure 2).

For the platelet reactivity assay, single platelets were gated (Figure 1A), and both P-selectin expression and open GPIIbIIIa were determined based on MFI levels (Figure 1C). To validate the use of the platelet reactivity assay for the determination of platelet function in samples with low platelet numbers, platelet reactivity was measured in PRP samples of healthy controls diluted to a platelet number of 250, 50, 25, and 10×10^9/L. Dilution was accomplished by addition of plasma, so that only platelet count was diluted without dilution of plasma proteins. Platelet reactivity within donors, quantified by agonist concentration needed to obtain half maximal activation (EC50) and by response to maximal agonist concentration, did not change when platelet number was reduced to 10×10^9/L (supplemental Figure 3). Although validation experiments already showed that parameters of platelet reactivity were not dependent on platelet number, we investigated if platelet count possibly affected platelet reactivity in our study population. In the 33 patients, no correlation was observed between platelet number at day of inclusion and platelet reactivity ($\rho = 0.165$; $P = .367$; supplemental Figure 4).

Microaggregation of platelets

To assess whether the test correctly identified patients with known functional platelet defects, an acquired and a primary Glanzmann patient were tested. Baseline levels were completely normal (Figure 2A). Aggregation upon stimulation with ristocetin via GPIbIX was normal in the acquired Glanzmann patient, whereas the primary Glanzmann patient reached approximately half that of the healthy control (Figure 2B). As expected, almost complete inhibition of platelet aggregation was observed in both patients when stimulated with PMA via GPIIbIIIa (Figure 2C).

We subsequently performed the platelet microaggregation test on 18 chronic ITP patients, 11 with mild and 7 with severe bleeding phenotype. Unstimulated platelets of mild and severe phenotype patients did not aggregate over time (Figure 2D,G). After stimulation with ristocetin, mild phenotype patients equaled their healthy control after both 5 (47.6% [25.2-58.1], 49.9% [33.0-56.9]; $P = .971$) and 10 minutes (53.8% [37.9-65.7], 54.3% [41.4-71.2]; $P = .684$) (Figure 2E). Whereas severe phenotype patients tended to have lower aggregation levels after 5 (27.1% [24.0-41.7]) and 10 minutes (35.3% [29.2-52.7]) compared with healthy controls (49.2% [31.5-55.7], $P = .073$; 55.8% [40.9-66.2], $P = .125$), the differences were not significant (Figure 2H). Upon stimulation with PMA, mild phenotype patients equaled their healthy control after 5 (45.2% [34.7-70.0], 63.0% [47.3-70.9]; $P = .481$) and 10 minutes (71.9% [50.4-84.9], 80.6% [62.2-85.83]; $P = .631$) (Figure 2F). In contrast, severe phenotype patients had significantly lower aggregation levels after 5 (30.9% [16.0-42.8]) and 10 minutes (52.4% [28.7-66.45]) of
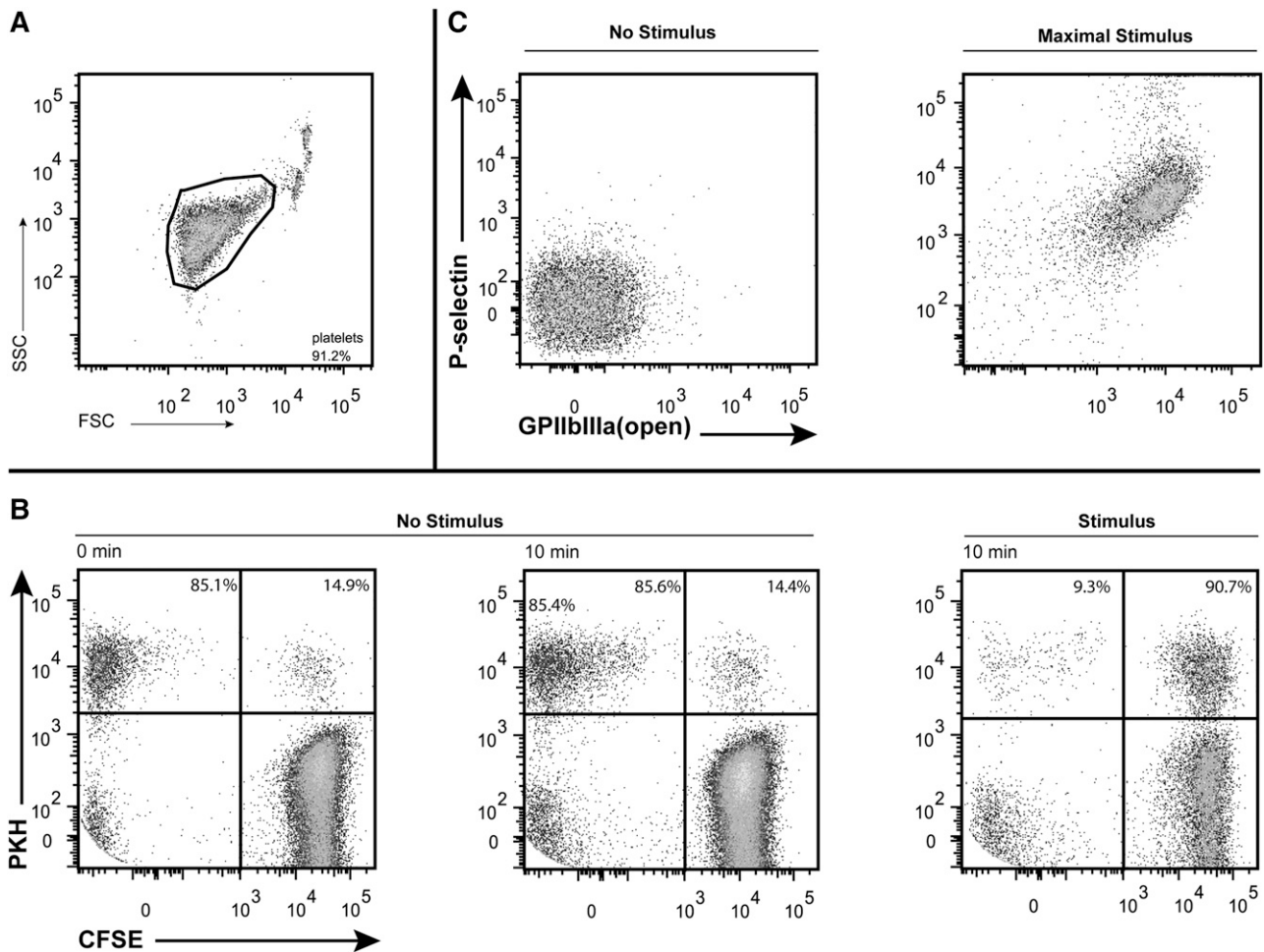
Figure 1. Methods for platelet microaggregation test and platelet reactivity assay. (A) Platelets were gated based on FSC/SSC properties. (B) Platelet microaggregation test. Test platelets, derived from either a patient or healthy control were stained with PKH and mixed in a 1:9 ratio with healthy control platelets, stained with CFSE. Platelets were analyzed in the phycoerythrin (PE; PKH) and fluorescein isothiocyanate (CFSE) channels; platelets positive in both channels were identified as aggregates. The left panel shows unstimulated platelets after 0 minutes, the middle panel unstimulated platelets after 10 minutes, and the right panel PMA-stimulated platelets after 10 minutes. (C) Platelet reactivity assay. The MFI of RPE-labeled mouse anti-human P-selectin antibodies and Alexa Fluor 488–labeled fibrinogen on platelets was measured by FACS analysis, representing the quantity of P-selectin and open GPIIbIIIa receptor per platelet. The left panel shows unstimulated platelets; the right panel shows platelets stimulated with 625 μM TRAP.

PMA stimulation compared with healthy control platelets (64.2% [51.4-65.2], $P = .021$; 80.8% [67.3-80.9], $P = .017$) after 5 and 10 minutes, respectively (Figure 2I).

Platelet reactivity assay

The basal level of platelet activation and platelet reactivity to the agonists ADP, CVX, and TRAP was determined for all included patients by measuring platelet P-selectin expression and opening of the GPIIbIIIa receptor in whole blood (Figure 3). There were no significant differences when comparing patients with chronic ITP with a mild phenotype to healthy controls.

Basal levels of platelet activation did not differ between chronic ITP patients with a mild (n = 23) and with a severe bleeding phenotype (n = 10), nor when compared with healthy controls (n = 8) (Figure 3A-B). Platelet degranulation, determined via measurement of P-selectin expression, in response to maximal ADP stimulation was decreased in patients with a severe bleeding phenotype (9.6×10^2 AU [3.7-11.8]), compared with both patients with a mild phenotype (13.0×10^2 AU [11.2-15.3]; $P = .020$) and controls (22.4×10^2 AU [10.7-25.5]; $P = .016$) (Figure 3C). When stimulated with CVX or

TRAP, no differences in P-selectin expression were observed between mild and severe bleeders (Figure 3G,K-L). Patients with a severe phenotype did need more CVX (11.0 ng/mL [5.6-393.8]) to stimulate for half-maximal P-selectin expression compared with healthy controls (4.7 ng/mL [2.6-6.4]; $P = .021$) (Figure 3H).

The concentration of ADP and CVX needed to obtain opening of the half-maximal amount of GPIIbIIIa receptors was higher in severe bleeders (1.1 μM ADP [0.9-1.5] and 9.0 ng/mL CVX [5.2-12.9]), compared with both mild bleeders (0.7 μM ADP [0.4-1.0] and 5.8 ng/mL CVX [2.8-9.0]; $P = .031$ and .031) and healthy controls (0.5 μM ADP [0.4-0.9] and 3.0 ng/mL CVX [1.4-4.0]; $P = .027$ and .006) (Figure 3F,J). Reactivity to TRAP was equal between groups (Figure 3M-N). Maximal GPIIbIIIa response to all agonists did not differ between groups (Figure 3E,I,M).

Platelet microaggregation vs platelet reactivity

In both the microaggregation and platelet reactivity assay, the platelets from severe ITP patients showed diminished activity compared with healthy controls. Indeed, PMA-induced aggregation and maximal platelet P-selectin response to ADP, the parameters giving
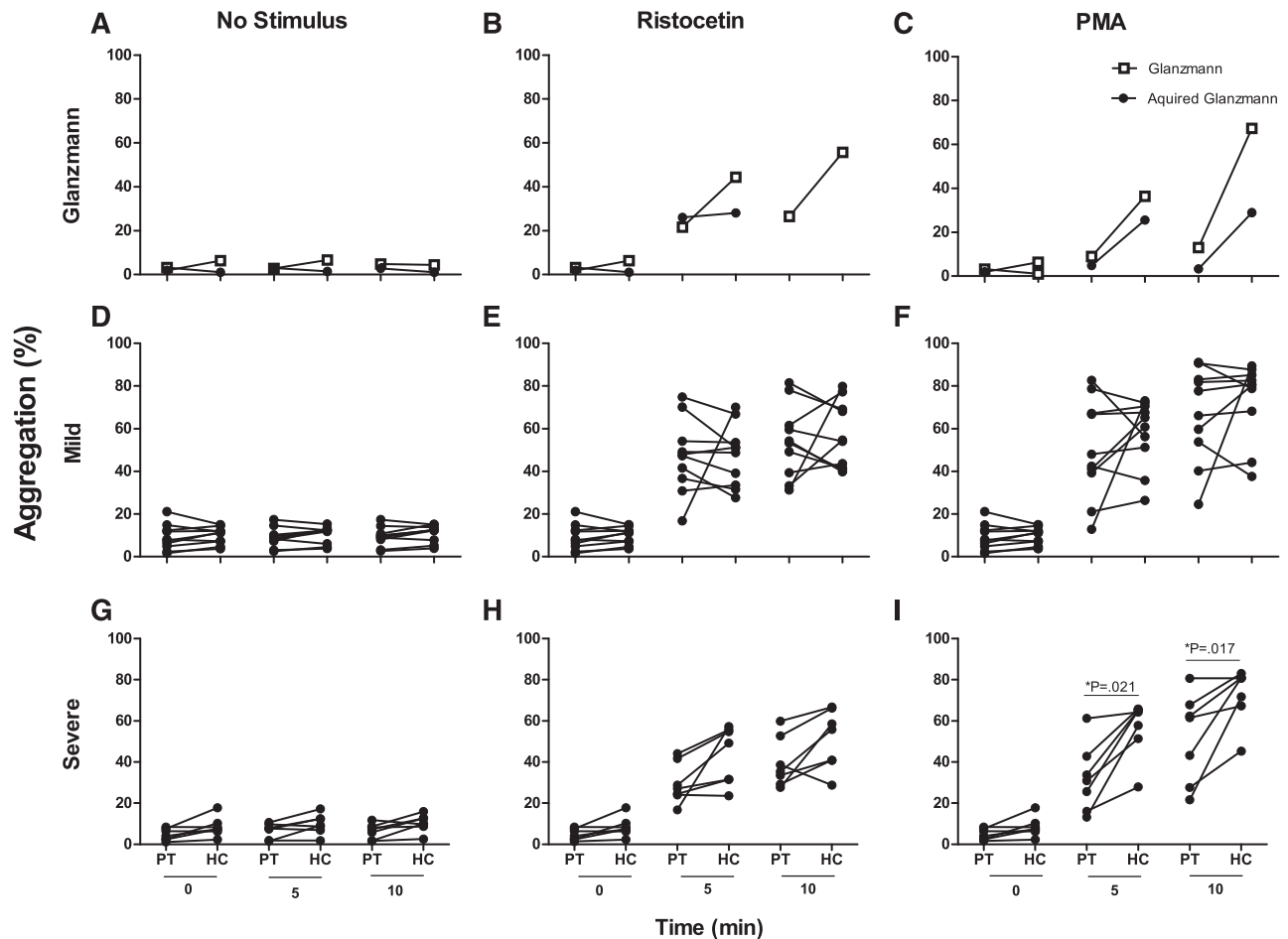
Figure 2. Severe chronic ITP patients show functional defects in platelet microaggregation upon PMA stimulation. An acquired Glanzmann patient, with functional GPIIbIIIa and an anti-GPIIbIIIa antibody, and a primary Glanzmann patient, without functional GPIIbIIIa, served as negative controls for the assay (A-C). Eleven mild chronic ITP patients (D-F) and 7 severe chronic ITP patients (G-I) were tested in the platelet microaggregation test. Results are given as percentage of aggregation reached after 0, 5, and 10 minutes without (A,D,G), after ristocetin (B,E,H), or after PMA stimulus (C,F,I). Outcomes are depicted pairwise, the patient compared with a healthy control tested in parallel. Statistical analysis was performed by Wilcoxon signed ranks testing.

the most discrimination between healthy controls and patients within the assays, correlated significantly for the 18 patients in which both assays were performed ($\rho = 0.519$; $P = .027$) (Figure 4). In supplemental Table 1, the characteristics of both assays are listed.

Discussion

A variation in bleeding severity exists between patients with chronic ITP that cannot be explained by platelet counts alone. We therefore hypothesized that platelet function might be affected diversely between patients with chronic ITP. In the current study, we observed a functional platelet defect in chronic ITP patients with a severe bleeding phenotype. These platelets displayed a decreased potential to form microaggregates following PMA stimulation, decreased platelet degranulation following ADP stimulation, and higher ADP and CVX concentrations needed for half-maximal activation of the GPIIbIIIa complex.

To our knowledge, this is the first study to establish platelet function in individuals with low platelet counts. Classical platelet function tests are not reliable with platelet counts below 50×10^9/L.[2,4] In this study, we present the microaggregation test and the platelet

reactivity assay for their use in samples containing low platelet numbers down to 10×10^9/L. We show that the microaggregation test can be performed in 10 mL of blood with this minimal platelet number to assess platelet function directly, as a pathway specific function. In the platelet reactivity assay, only 150 μL of whole blood was needed for all performed platelet reactivity measurements. The assay determines the reactivity of single platelets independently of platelet number. Only 3 quick handling steps, incubation, fixation and red cell lysis, are needed to perform the assay because platelet isolation is not required. The serial dilutions used for platelet activation can be stored at $-20°C$ for several weeks and can be used instantly when needed. The assay has a broad and quantitative detection range, measuring both platelet degranulation and GPIIbIIIa opening, and it distinguishes between multiple specific activation pathways. In the scope of this research, we decided to test pathways of specific interest in ITP, but naturally, each agonist of choice can be used in both assays. Both tests might be of great diagnostic value in a broad range of patients suffering from thrombocytopenia. In this study in children with chronic ITP, we proved that we can determine platelet function and correlate this with bleeding phenotype.

We classified patients as having a mild or a severe bleeding phenotype using the overall Buchanan bleeding score.[16] We
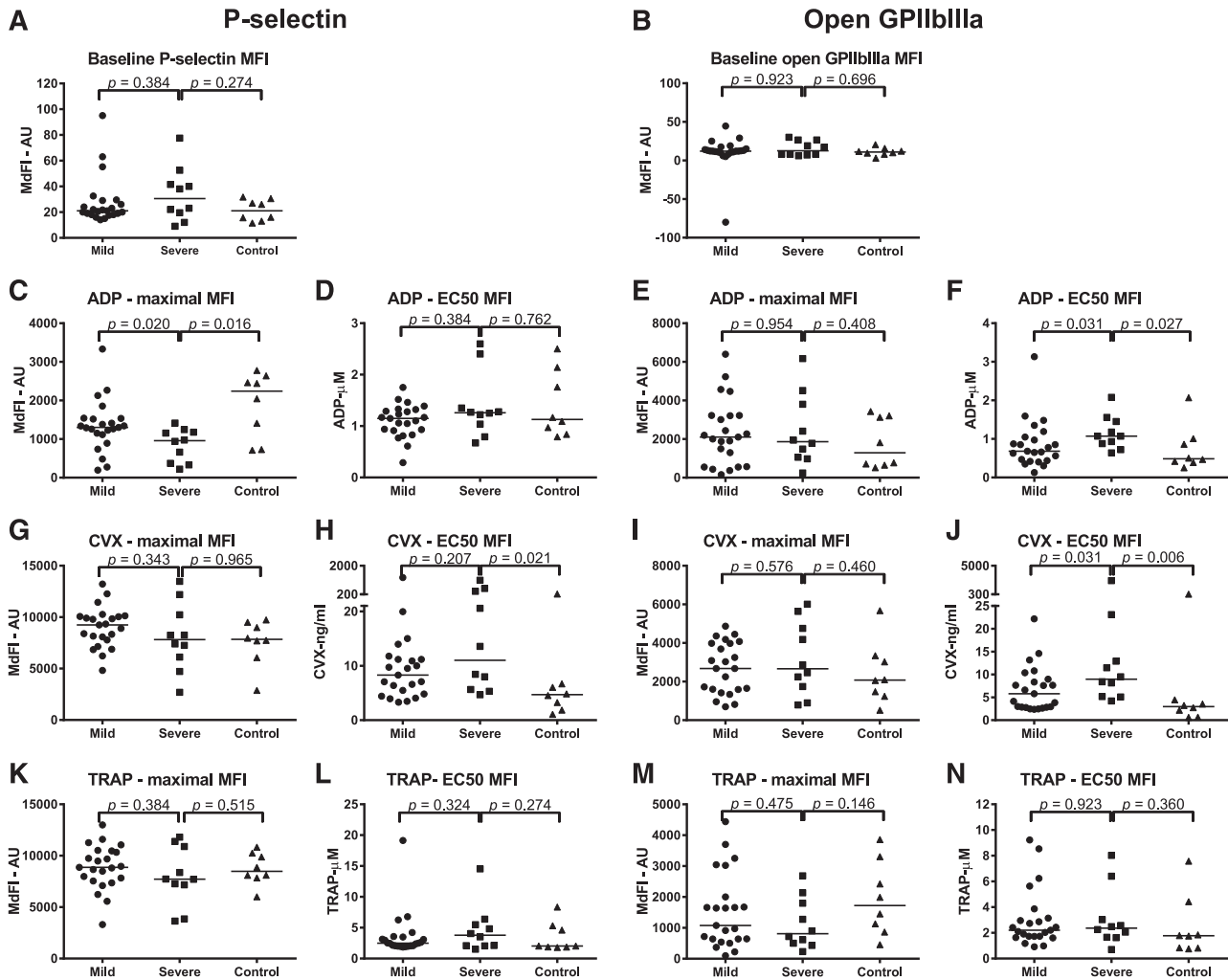
Figure 3. Severe chronic ITP patients show functional defects in platelet reactivity upon ADP and CVX stimulation. To determine baseline platelet reactivity, MFI in arbitrary units (AU) of fluorescent-labeled anti–P-selectin antibody (A) and of fluorescent-labeled fibrinogen (B) bound to platelets was determined in the absence of agonists. Maximal MFI in AU in response to stimulation with ADP, CVX, and TRAP and concentration of these agonists needed to obtain half-maximal MFI were determined for both platelet P-selectin expression (C-D,G-H,K-L) and opening of GPIIbIIIa receptor (E-F,I-J,M-N). The dots represent individual results for the 10 severe patients, 23 mild patients, and 8 healthy controls, with a line at the median. Statistical analysis was performed by Mann-Whitney *U* testing.

compared patients with a mild bleeding phenotype scoring a grade 0 to 3 to patients with a severe phenotype scoring a grade 4 to 5 in our search for a relation between platelet function and bleeding phenotype. This established score for ITP patients, which grades bleeding

Figure 4. Correlation of platelet microaggregation and platelet reactivity. Response to PMA-induced platelet microaggregation and ADP-induced maximal platelet reactivity correlated with a Spearman correlation coefficient of 0.519 (*P* = .027) for all 18 patients in which both assays were performed.

severity based on skin and mucosal bleeds, was demonstrated to be associated with platelet function.

With the microaggregation assay, we found patients classified as severe bleeders to have significantly lower aggregation levels compared with healthy controls upon 5 and 10 minutes of PMA stimulation. However, when stimulating with ristocetin, no differences between patients and healthy controls were observed. A possible explanation for the decreased PMA response might be an interference of autoantibodies present on patient platelets not detectable by indirect MAIPA or indirect PIFT because both assays have very low sensitivities (25% to 39% and 30%, respectively).[18-20] The presence of anti-GPIIbIIIa antibodies in these patients might explain why we predominantly see an effect with PMA stimulation and not with ristocetin, as these autoantibodies are most frequently found in ITP patients.[7,21] With the platelet reactivity assay, we found the severe patients to have significantly lower P-selectin expression after activation with ADP, but not after activation with CVX or TRAP. Next to platelet P-selectin expression, opening of the platelet GPIIbIIIa receptor was measured. We observed that for both stimulation with ADP and CVX, higher agonist concentrations were needed in patients with a severe phenotype to obtain opening of
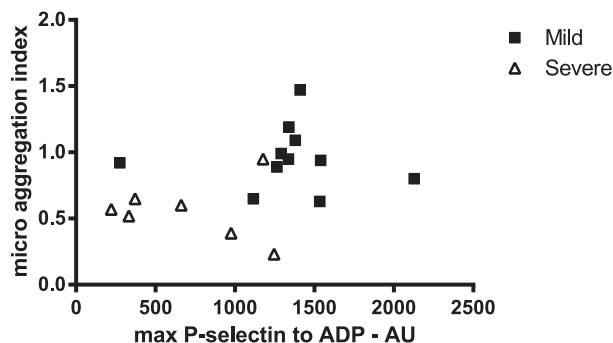
the half-maximal amount of GPIIbIIIa receptors, when compared both with mild patients and controls. Interference of autoantibodies in binding of fluorescein isothiocyanate–labeled fibrinogen to open GPIIbIIIa in the platelet reactivity assay seems unlikely because differences in GPIIbIIIa opening are seen only for ADP and CVX stimulation, and not following TRAP stimulation. Theoretically, the decrease in platelet reactivity observed in severe patients might be caused by an interaction of autoantibodies with platelets or mega-karyocytes, leading to outside-in signaling influencing platelet reac-tivity to natural stimuli or influencing megakaryocyte maturation and differentiation.

A limitation to our study is that we cannot confirm if the decreased platelet activity that we observed in the more severe ITP patients was attributable to anti-platelet antibodies. Although direct variants of PIFT and MAIPA are more sensitive, these still require a high number of platelets and are therefore unsuitable for severe cases with a low number of platelets.[18,20] Therefore, direct confirmation of whether autoantibodies are to blame will have to wait until more reliable methods are developed to detect low levels of anti-platelet antibodies using a low number of platelets. Also, a larger prospective study determining platelet function, and estimating bleeding tendency with the new ITP bleeding assessment tool, will be required to determine if the assay reported here can predict severity.[22]

Within healthy adult individuals, platelet parameters are known to be stable when measured at several time points.[23] A single measurement represents a person's innate platelet reactivity, making repeated measurements unnecessary. However, we cannot exclude antibodies responsible for ITP to influence this innate platelet reactivity via outside-in signaling. Platelet reactivity in chronic ITP patients could therefore vary along with disease activity. Future longitudinal studies will be necessary to determine whether platelet reactivity is stable in chronic ITP.

In this study, we have focused on in vitro platelet function in the context of bleeding severity in chronic ITP. Nevertheless, in vivo other factors could influence bleeding severity as well, such as endothelial function and plasma factors. These factors might, to-gether with platelet function, influence bleeding phenotype.

In summary, here we have established 2 functional tests that allow for evaluation of platelet function in children with chronic ITP and enable us to associate the results with the bleeding phenotype. Patients with a severe bleeding phenotype were found to have a decreased platelet function, shown by decreased platelet aggregation following PMA stimulation in the platelet microaggregation test, decreased platelet degranulation following ADP stimulation in the platelet reactivity assay, and higher ADP and CVX concentrations needed for half-maximal activation in the platelet reactivity assay. Longitudinal studies will have to confirm if the microaggregation test and the platelet reactivity assay can be used to predict bleeding phenotype in chronic ITP.

Acknowledgments

Authorship

Contribution: E.R.v.B., A.G.L., K.M.H.-P., L.B.v.d.H., P.G.d.G., G.V., and M.C.A.B. designed the study; E.R.v.B., L.B.v.d.H., M.R., and P.G.d.G. were responsible for the experimental design of the platelet reactivity assay; A.G.L., L.P., C.E.v.d.S., M.d.H., and G.V. were responsible for the experimental design of the platelet micro-aggregation assay; E.R.v.B. and L.B.v.d.H. performed all platelet reactivity assays; A.G.L. performed all platelet microaggregation tests; E.R.v.B. and A.G.L. carried out the data analysis; E.R.v.B., A.G.L., L.B.v.d.H., P.G.d.G., G.V., and M.C.A.B. wrote the manuscript; and all authors contributed to data interpretation, contributed to revising the manuscript for intellectual content and style, and approved the final version.

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